J Appl Biotechnol Rep. 2023 September;10(3):1069-1078

Journal of
Applied Biotechnology Reports

doi 10.30491/JABR.2022.345443.1540

Original Article

Establishment and Characterization of Melanocyte Stem Cells from Hair Follicle Bulge of Vitiligo Patients

Manchi Vathsalya [1], Shricharith Shetty [2], Nikhil Shetty [3], Shama Rao [1], Veena Shetty [1], Sunil Kumar Yeshwanth [4], Mohana Kumar Basavarajappa [1*]

[1] Nitte University Centre for Stem Cell Research and Regenerative Medicine, K. S. Hegde Medical Academy, Nitte (Deemed to be University), Deralakatte-575018, Mangaluru, India
[2] Department of Dermatology, K. S. Hegde Medical Academy, Nitte (Deemed to be University), Deralakatte-575018, Mangaluru, India
[3] Department of Plastic Surgery, K. S. Hegde Medical Academy, Nitte (Deemed to be University), Deralakatte-575018, Mangaluru, India
[4] Department of Pathology, K. S. Hegde Medical Academy, Nitte (Deemed to be University), Deralakatte-575018, Mangaluru, India

Corresponding Author: Mohana Kumar Basavarajappa, PhD, Associate Professor, Nitte University Centre for Stem Cell Research and Regenerative Medicine, K. S. Hegde Medical Academy, Nitte (Deemed to be University), Deralakatte-575018, Mangaluru, India. Tel: +91-9480504462, E-mail: mohanakumar@niite.edu.in

Received June 3, 2022; **Accepted** December 21, 2022; **Online Published** September 10, 2023

Abstract

Introduction: Melanocyte stem cells (MelSCs) might offer potential candidates for cell-based therapies against pigmentary disorders, such as vitiligo. However, the properties of MelSCs and their involvement in disease pathology are yet to be explored. In this study, MelSCs derived from the lesional (V-MelSCs) and unaffected regions of vitiligo patients (NV-MelSCs) were established, and compared their cellular, biological and molecular characteristics to that of MelSCs isolated from the skin of normal, unaffected individuals as control (C-MelSCs).

Materials and Methods: MelSCs were isolated and expanded *in vitro* by explant culture. Growth kinetics, metabolic activity, cell viability, colony-forming ability, senescence activity, and cell cycle status of MelSCs were analyzed. Later, MelSCs were evaluated for the expression of marker genes, such as *CD34, Pax3, Sox10, sFRP4, MITF, TYRP1, DCT, TYR, TGFα,* and *c-KIT*.

Results: MelSCs derived from vitiligo patients had lower proliferation and metabolic activity despite showing unaltered morphological features. Significantly (P<0.05) decreased number of viable cells was observed in C-MelSCs and V-MelSCs in passage 3 (P3) compared to P1. The colony-forming assay revealed homogeneous distribution, but slightly larger and dense colonies in C-MelSCs compared to NV-MelSCs and V-MelSCs. All MelSCs showed a higher percentage in the G0/G1 phase of the cell cycle with low senescence activity. Finally, MelSCs from the skin of vitiligo exhibited lower expression levels of selected marker genes implicated in melanocyte development.

Conclusions: MelSCs residing in hair follicle bulge of vitiligo patients possess marginally altered cellular, potency and biological characteristics. These findings underscore the challenges in establishing repigmentation therapy for vitiligo using autologous transplantation of MelSCs.

Keywords: Hair Follicle Bulge, Melanocyte Stem Cells, Vitiligo, *In Vitro*, Comparative Characterization

Citation: Vathsalya M, Shetty S, Shetty N, Rao S, Shetty V, Yeshwanth SK, et al. Establishment and Characterization of Melanocyte Stem Cells from Hair Follicle Bulge of Vitiligo Patients. J Appl Biotechnol Rep. 2023;10(3):1069-1078. doi:10.30491/JABR.2022.345443.1540

Introduction

Hair follicle stem cells (HFSCs), which reside in the bulge region, are responsible for giving rise to cell types of hair and also the epidermis and sebaceous glands.[1] Melanocyte stem cells (MelSCs), a distinct population of HFSCs, are involved in the pigmentation of the newly formed hair and the skin epidermis. They remain quiescent until they get activated at the new growth phase of the hair cycle (anagen) and proliferate to produce melanocyte progenitor cells.[2] The cells migrate towards the hair bulb to eventually differentiate into mature melanocytes. As the regression phase of the hair cycle (catagen) begins, differentiated mature melanocytes undergo apoptosis, while MelSCs in the follicle bulge survive. Consequently, during the rest phase of the hair cycle (telogen), MelSCs remain quiescent until they are reactivated.[3]

Vitiligo is the prevalent pigmentation disorder characterized by patchy loss of skin pigment, which is understood to have autoimmune causation. Infiltrates of activated cytotoxic $CD8^+$ T-cells and macrophages have been detected bordering the unstable vitiligo lesions, progressively destroying mature epidermal melanocytes.[4,5] Most current therapeutic regimes for vitiligo are focused on 'repigmentation', which refers to the restoration of pigment by stimulating melanocytes and their precursors or suppressing immune response in the lesional areas. These treatments include non-invasive and surgical techniques. Ultraviolet radiation therapy and topical steroids are the most common non-invasive techniques. However, response to non-invasive treatments is typically inadequate and incomplete. Therefore, surgical procedures, such as tissue and cellular grafting, have been employed to achieve the desired degree of repigmentation. Cellular transplantation typically involves autologous cultured melanocyte

suspension or non-cultured epidermal cellular suspensions.[6-9] Although this procedure has reported successful repigmentation in patients, it does pose limitations regarding the size of the donor site for larger lesional areas as well as the extended culture time to obtain a substantial number of cells.

MelSCs are potential candidates for cell-based therapies against pigmentary disorders owing to their self-renewal, multipotent and migratory properties. MelSCs have been isolated and studied in rodents as well as in zebrafish embryos.[10-14] Due to a lack of specific identifying molecular markers and established isolation protocols, MelSC cultures from human hair follicles have not yet been accomplished.[15] In this *in vitro* study, MelSCs derived from the lesional and unaffected regions of vitiligo patients were established and compared on their cellular, biological and molecular characteristics to that of MelSCs isolated from the skin of normal, unaffected individuals.

Materials and Methods

Sample Collection

The patients admitted with stable vitiligo (n = 2) as well as with no inflammatory skin disorders designated as controls (n = 2) were selected. Punch biopsy of unshaved skin from the affected and unaffected regions of vitiligo patients and full thickness skin biopsy from normal patients were collected. Samples were obtained after ethics committee approval and informed consent was taken in accordance with the Declaration of Helsinki.

Isolation and Culture Expansion of MelSCs

Skin biopsy was washed with Dulbecco's phosphate-buffered saline (DPBS, GIBCO, Grand Island, NY, USA) containing 100 U/ml penicillin, and 100 µg/ml streptomycin, and tissue pieces were digested in 0.5% dispase (Himedia, Mumbai, India) for 30 min in an incubator-shaker followed by 0.25% trypsin (Himedia) for 30 min. The separated hair follicles were placed as explants on 0.1% gelatin-coated culture plates. Minimum essential medium (MEM-α, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO, Brazil, South America), 0.5% human melanocyte growth supplement (HMGS, GIBCO) and 2 ng/ml epidermal growth factor (EGF, Biolegend, San Diego, CA, USA) was used to support the initial release of cells. The media was changed every third day and culture plates were incubated at 37 ºC with 5% CO_2 in the air.

Adherent cells from primary culture (P0) were passaged using 0.25% trypsin-EDTA upon reaching 90% confluence. Cells were then cultured in MEM-α supplemented with 5% FBS, 0.5% HMGS, and 2 ng/ml EGF with a media change every third day. After reaching confluency at passage 1 (P1), part of the cells was sub-cultured (P2) and the rest was stored at -80 ºC. The workflow of isolation and establishment of MelSC cultures *in vitro* has been presented schematically in Figure 1 (created using Biorender.com).

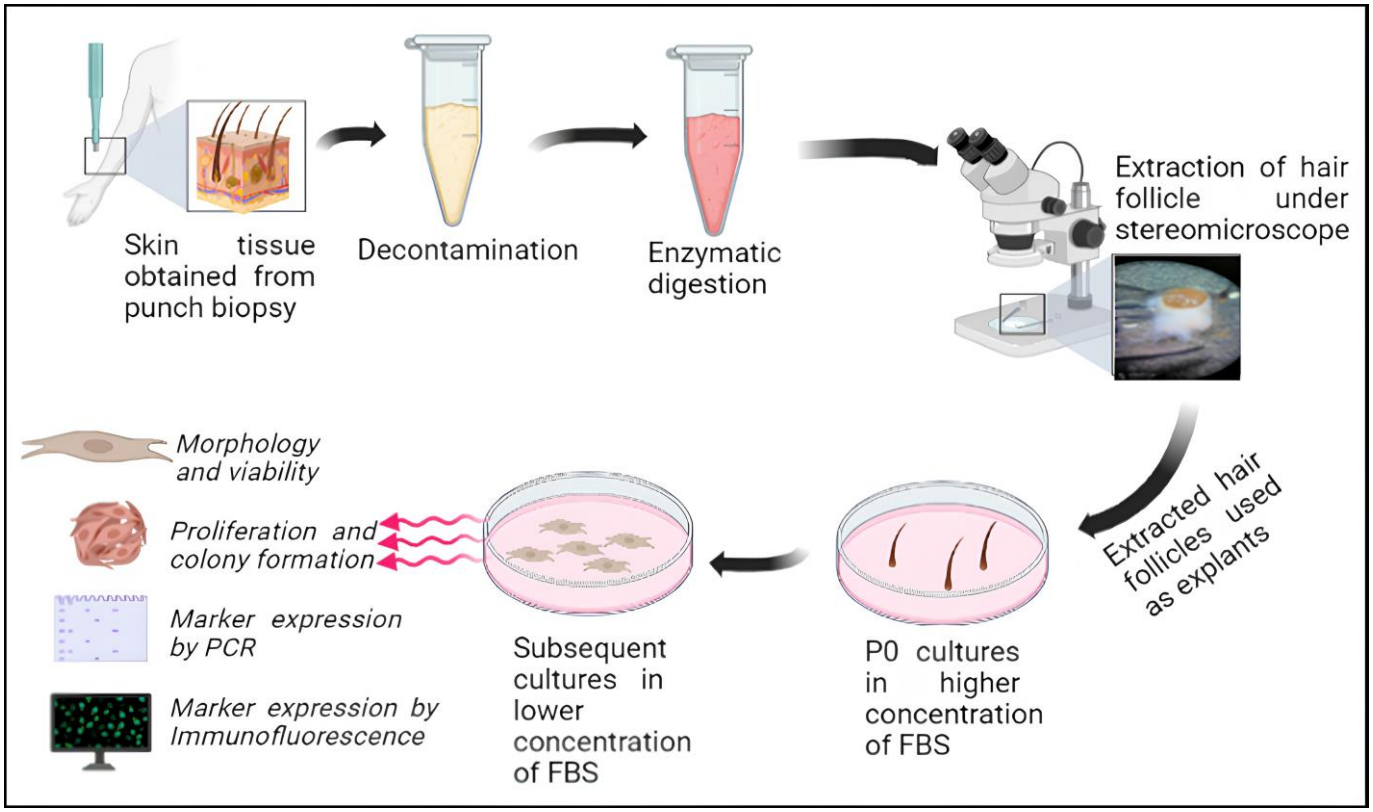

Figure 1. Schematic Representation of the Study Workflow (created using Biorender.com).

Assessment of Morphology and Viability

Morphology of MelSCs at each passage was observed using a phase-contrast microscope (Olympus, Tokyo, Japan). Cell viability was assessed at each passage using 0.4% trypan blue exclusion test by counting the cells using a haemocytometer.

Evaluation of Growth Characteristics

Cell proliferation rate at P3 was evaluated by seeding 5×10^3 cells per well of a 12-well plate (Thermo Scientific, Roskilde, Denmark). Every third day, cells were detached and counted on a haemocytometer. Similarly, population doubling time (PDT) was calculated using the formula PDT = t (log2)/(log Nt-log No), where t represents culture time, and No and Nt are the cell numbers before and after seeding, respectively.

MTT Assay

A methyl triazolyl tetrazolium (MTT, Himedia)-based assay was performed to evaluate the cell metabolic activity. About 5×10^3 cells were seeded in each well of a 24-well plate (Thermo Scientific) and cultured for 12 days. Every third day, cells were incubated with 300 µl of 0.5 mg/ml of MTT solution for 3 h at 37 ºC following which 300 µl of dimethyl sulfoxide was added with further incubation of 15 min. 100 µl of this solution was then transferred to a 96-well plate and absorbance was measured.

Colony-formation Assay

MelSCs were assessed for the ability of colony formation by seeding 2×10^3 cells in a 100 mm culture dish (Thermo Scientific). The media was changed every three days. On day 14, the colonies were fixed in 3.7% formaldehyde (Sigma-Aldrich) for 20 min and stained with 1% crystal violet solution (Sigma-Aldrich). After 4 h, the stain was removed and the images of colonies were captured.

Cell Cycle Analysis

MelSCs (0.5×10^6) were fixed with 70% ethyl alcohol (Sigma-Aldrich), and washed twice with DPBS, prior to incubation with 100 mg/ml RNase (Sigma-Aldrich) at 37 ºC for 1 h. 1 mg/ml propidium iodide (PI, Life Technologies, CA, USA) was then used as a DNA stain and allowed for 1 h at room temperature in the dark. The cell cycle was assessed using a flow cytometer (PARTEC CyFlow, Goerlitz, Germany).

Senescence Associated β-galactosidase (SA-β-Gal) Assay

The senescence activity in MelSCs at P4 was assessed using the SA-β-Gal staining kit (Himedia) following manufacturers' instructions. Briefly, DPBS-washed cells were fixed for 15 min in 3.7% formaldehyde (Sigma-Aldrich), and the colour development was observed by overnight incubation at 37 °C with kit-supplied SA-β-Gal staining solution. The development of blue colour was observed under the phase-contrast microscope.

Reverse Transcription-polymerase Chain Reaction (RT-PCR) and Real Time-Quantitative PCR (qPCR) Analysis

Total RNA was extracted from cells using the RNAiso Plus kit (Takara, Tokyo, Japan). Complementary DNA (cDNA) was synthesized using PrimeScriptTM RT reagent kit (Tak,ara) and PCR amplification was performed with EmeraldAmp® GT PCR Mastermix (Takara). The PCR products were fractionated by 1% agarose gel electrophoresis. Details of primers used are presented in Table 1. qPCR was performed using SYBR™ Green master mix (Thermo Fisher Scientific) on StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The quantification of selected genes, such as *CD34*, *Pax3*, *Sox10*, *sFRP4*, *MITF*, *TYRP1*, *DCT*, *TYR*, *TGFα*, and *c-KIT* was carried out and *ß-actin* was employed for normalization. The CT values of

Table 1. List of Primer Sequences

Gene	Sequence (5'-3')	Accession number	Base pair (bp)
β-ACTIN -F	TCCTTCCTGGGCATGGAG	NM_001101.2	207
β-ACTIN -R	AGGAGGAGCAATGATCTTGATCTT		
CD34 - F	AGACTGTGCAGTGATGTGGT	NM_001025109	174
CD34 - R	CTGGTACATTCGGGTCTGCC		
Pax3 - F	GTGTCTCTGCGCCTGAGTAA	NM_000438.6	246
Pax3 - R	TCTATCCCCGGCCCTACAAT		
Sox10 - F	CACAAGAAAGACCACCCGGA	NM_006941.4	146
Sox10 - R	AAGTGGGCGCTCTTGTAGTG		
sFRP4 - F	GCGGAGAACAGTTCAGGACA	NM_003014.4	193
sFRP4 - R	AGTCGGAAGTCTCCGCTTTG		
MITF - F	GCCTGTCTCGGGAAACTTGA	NM_000248.4	138
MITF - R	GCCAGTGCTCTTGCTTCAGA		
TYRP1 - F	GTGCCACTGTTGAGGCTTTG	NM_000550.3	155
TYRP1 - R	ATGGGGATACTGAGGGCTGT		
DCT -F	TCTTTTGGGCACTCAGCATGTA	NM_001129889.3	185
DCT - R	CTTCACCTGAGGAAAGGCTGG		
TYR -F	CTTGTGAGCTTGCTGTGTCG	NM_000372.5	150
TYR –R	GTGAGGTCAGGCTTTTTGGC		
TGFα -F	GGCTTCTTCAGGACAGCACT	NM_003236.4	126
TGFα -R	CCCAAGCAGACGGAGTTCTT		
C-Kit -F	GCTCTGCTTCTGTACTGCCA	NM_000222.3	212
C-Kit -R	GGTGTGGGGATGGATTTGCT		

control samples were used as calibrators to calculate relative quantification ($2^{-\Delta\Delta CT}$) of MelSCs-specific genes in vitiligo samples.

Immunofluorescence Assay of Selected Markers in MelSCs

MelSCs were examined for the expression of markers, such as CD34, MITF, and TYRP1 by immunofluorescence staining. Cells were seeded at a density of 5×10^3/well on a chamber slide and fixed with 3.7% paraformaldehyde (Himedia) after reaching 70% confluence. Post-treatment with 0.1% Triton X (Sigma-Aldrich) and 1% bovine serum albumin (BSA, Sigma-Aldrich), 200 µl of primary antibodies against CD34, MITF, and TYRP1 (Biolegend) were added to the respective wells and incubated overnight. Cells were then incubated in fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) for 1 h, followed by PI (Himedia) counterstain for 15 min. Coverslip was then placed on the slide and imaging was performed using a fluorescence microscope (Nikon Eclipse Ni-U, Tokyo, Japan).

Statistical Analysis

One-way or two-way analysis of variance (ANOVA) followed by Tukey's post-test was used to compare differences (Graph Pad Prism software, Version 8). All experiments were performed in triplicates. Data are presented as mean ± standard deviation (SD). *P* value<0.05 was considered statistically significant.

Results

Morphology

A heterogeneous population of highly adherent cells was obtained in the primary cultures (P0) with hair follicles as explants (Figure 2). The initial release of cells from control samples (C-MelSCs) needed 7-10 days, whereas, it required 15 days in cell cultures from non-vitiligo (NV-MelSCs) and vitiligo (V-MelSCs) regions of patients. Squamous cells bordered the hair follicle bulge, which was surrounded by spindle-shaped cells with dendritic features in primary cultures (P0). Squamous cells exhibited low adherence in subsequent passages, and a higher number of dendritic cells was obtained in P1 and P2. Collectively, no noticeable differences in morphology were observed between the hair follicle samples of controls and vitiligo cell cultures.

Cell Viability

The viability of MelSCs at every passage was consistently above 90% in all the cell lines. However, a significant (P<0.05) decrease in viable cells was observed in C-MelSCs and V-MelSCs in P3 compared to P1 (Figure 3A).

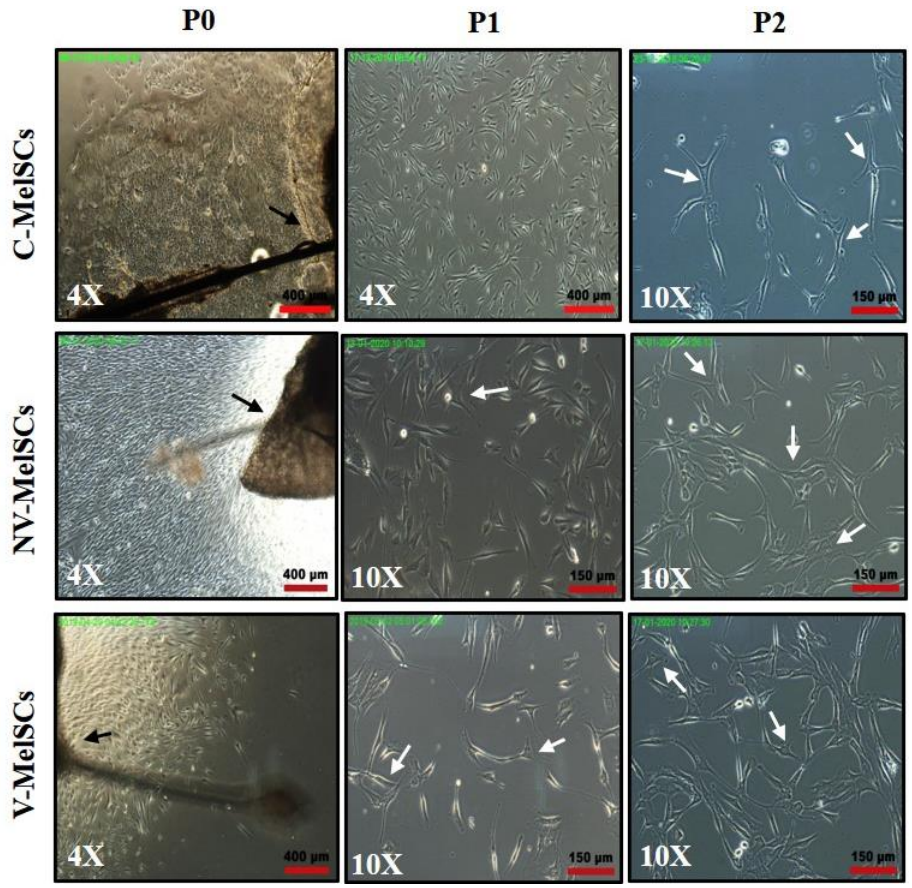

Figure 2. Isolation and Expansion of MelSCs from Human Hair Follicle Bulge. Cells in primary cultures (P0), passages 1 and 2 (P1 & P2) exhibited bi-, tri-, and multi-dendritic cells (arrows) with a prominent nucleus in all the groups.

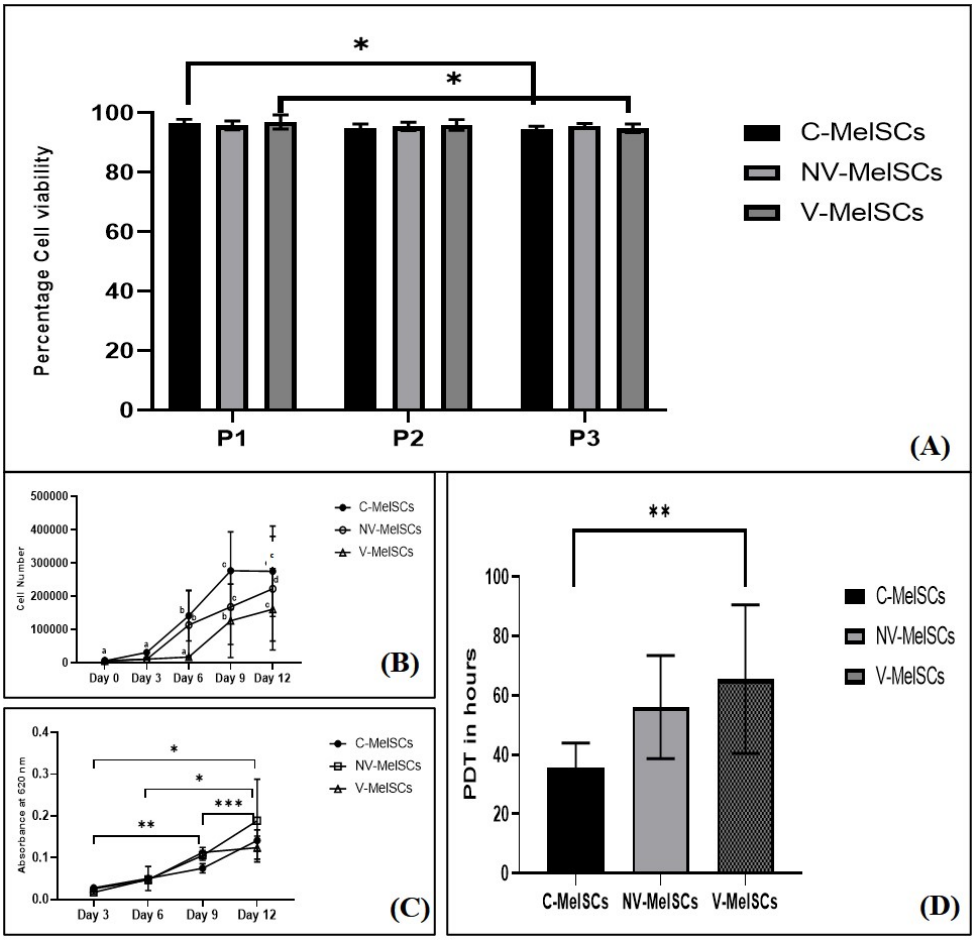

Figure 3. Assessment of Viability, Growth Kinetics, and Metabolic Activity of MelSCs. **(A)** Percentage viability of MelSCs at passage 1 (P1), P2, and P3. * indicates significant differences at $P<0.05$; **(B)** Proliferation rate of MelSCs at different time intervals. a, b, and c indicates significant differences at $P<0.05$; **(C)** MTT assay for the assessment of cellular metabolic activity. *Significant increase in metabolic activity in cells in all the groups. **NV-MelSCs and V-MelSCs showed significantly increased activity. ***NV-MelSCs alone exhibited increased metabolic activity; **(D)** PDT of MelSCs in hours. ** indicates a significant difference at $P<0.05$.

Growth Kinetics and Metabolic Activity

The proliferation rate of MelSCs from control had a faster growth rate than vitiligo (Figure 3B). The number of cells did not increase significantly ($P>0.05$) from day 0 to 3 in all the groups. C-MelSCs and NV-MelSCs showed a significant ($P<0.05$) increase in proliferation from day 3 to 6 and day 6 to 9. C-MelSCs attained confluence on day 9 and did not show any increase in a number of cells from day 9 to 12, whereas NV-MelSCs continued to proliferate after day 9. V-MelSCs showed slow proliferation until day 6, but later exhibited increased number. The average PDT of C-MelSCs was 34 h, and it was significantly lower than V-MelSCs with an average PDT of 65 h (Figure 3D). NV-MelSCs with a mean PDT of 56 h did not differ significantly ($P>0.05$) from C-MelSCs.

MTT assay showed a linear increase in absorbance values when measured every three days in all the cell lines (Figure 3C). However, all the groups observed a significant ($P<0.05$) difference from day 6 to 12. Additionally, NV-MelSCs and V-MelSCs exhibited a significant ($P<0.05$) increase in absorbance from day 3 to 9 and day 9 to 12.

Colony-forming Assay

The colonies of MelSCs were observed both macroscopically and microscopically with variations in size and cell density by crystal violet staining (Figure 4A). The colonies formed were homogeneously distributed in all the cell lines, but slightly larger and dense colonies were observed in C-MelSCs compared to NV-MelSCs and V-MelSCs.

Cell Cycle Status

C-MelSCs exhibited 69 ± 0.08% of cells in the G0/G1 phase, whereas NV-MelSCs and V-MelSCs had 83.0 ± 0.01% cells (Figure 4B). Further, in C-MelSCs, S-phase had 13.5 ± 0.01%, and the G2/M phase showed 2.8 ± 0.01% of cells. NV-MelSCs exhibited 9.0 ± 0.02% of cells in the S-phase and 1.2 ± 0.01% cells in the G2/M phase. V-MelSCs presented 5.7 ± 0.06% and 1.4 ± 0.02% of cells in the S-phase and G2/M phase, respectively.

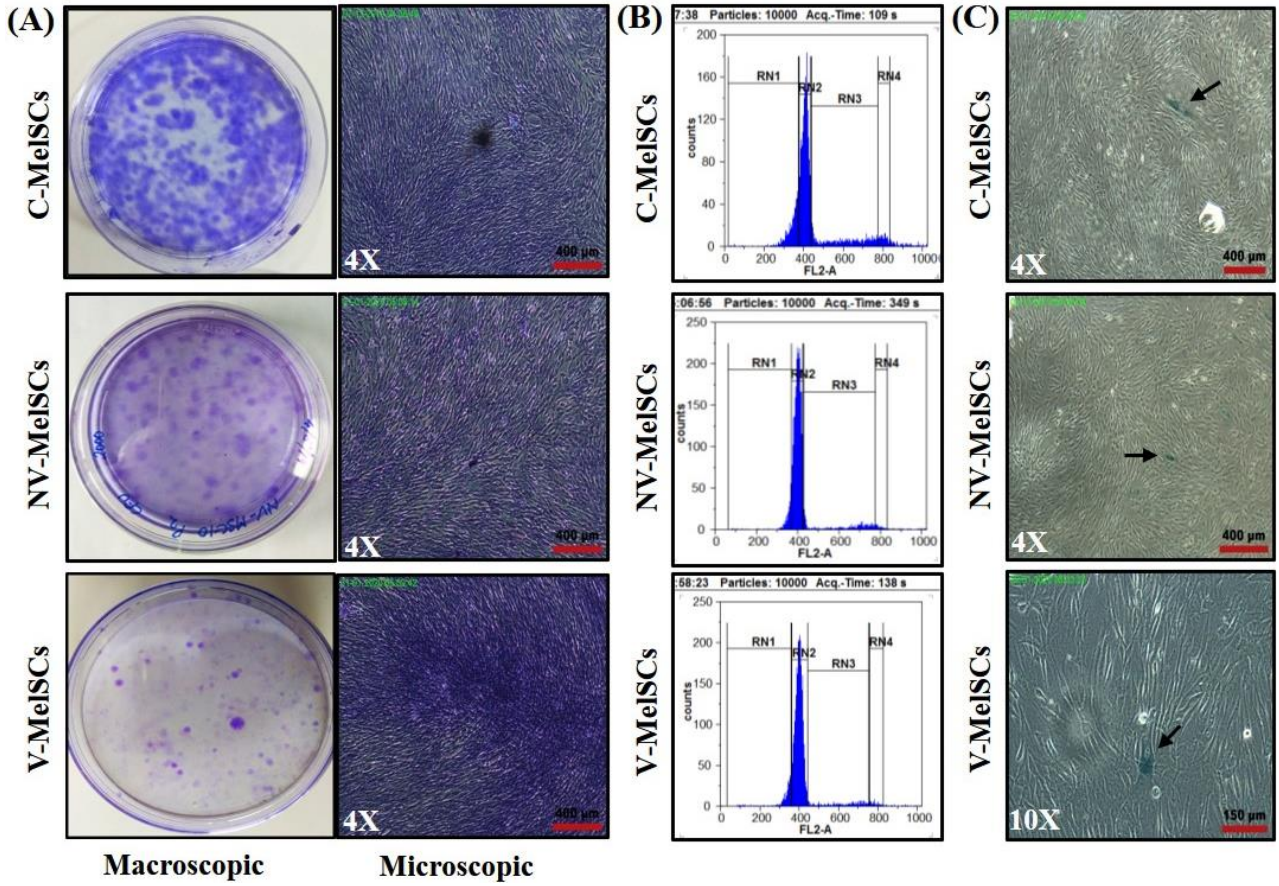

Figure 4. Colony-forming Ability, Cell Cycle Status, and Senescence Activity in MelSCs. **(A)** Colony-formation was observed in MelSCs cultured for 14 days with crystal violet staining; **(B)** Flow cytometry-based cell cycle analysis of MelSCs; **(C)** Assessment of cell senescence by SA β-Gal assay. At later passage, little or no SA-β-gal expression was detected in MelSCs (P4).

Senescence Assay

MelSCs were stained with SA-β-gal, and aging cells stained in blue reflected SA-β-gal expression. As anticipated, little or no expression was observed in MelSCs (Figure 4C), thus exhibiting a minimal positivity for senescence activity at P4.

Relative Expression of Selected Genes

Analysis of mRNA expression of MelSCs specific genes (*CD34*, *Pax3*, *Sox10*, *sFRP4*, *MITF*, *TYRP1*, *DCT*, *TYR*, *TGFα,* and *c-KIT*) was performed by RT-PCR and qPCR. PCR products of studied genes were detected in all the cells (Figure 5A). In qPCR, C-MelSCs displayed significantly (P<0.05) higher expression of *TYR* and *TGFα* compared to vitiligo groups, and also the expression level of *MITF* in C-MelSCs was significantly (P<0.05) higher than in V-MelSCs. The expression level of *TYRP1*, *DCT*, and *c-KIT* was higher in C-MelSCs than in vitiligo groups, but the difference was not significant (P>0.05). Similarly, the expression of *CD34*, *Pax3*, *Sox10*, and *sFRP4* did not differ significantly (P>0.05) between the MelSCs (Figure 5B).

Immunofluorescence Staining of Selected Markers

Expression of selected MelSCs specific markers, such as CD34, MITF, and TYRP1 was evaluated by immunofluorescence staining. Amongst the markers, the expression level of CD34 was high in all groups (Figure 6A). Further, MelSCs were also stained positive for MITF and TYRP1 (Figures 6B and 6C).

Discussion

The hair follicle is regarded as a mini-organ that involves neuroectodermal-mesodermal interaction. Several signaling pathways, such as Wnt, Notch, and Hedgehog commune to orchestrate the complex process of hair follicle morphogenesis and regular cycling.[1,3] During the induction phase, Wnt signaling directs the basal epithelial cells to form a placode. The hair placode extends into the dermis to form the elongated hair follicle structure.[2,6] HFSCs are derived from the asymmetric division of SOX9 positive basal epithelial cells in the placode periphery and remain quiescent in the bulge region until the next hair cycle.[16] The bulge region harbors a relative immune privilege, rendering MelSCs less affected by the autoimmune attack.[17,18] Vitiligo is conceivably caused by an autoimmune reaction, and several conventional treatments, such as immunosuppressors, help in the regeneration of pigment-producing melanocytes, which is believed to occur

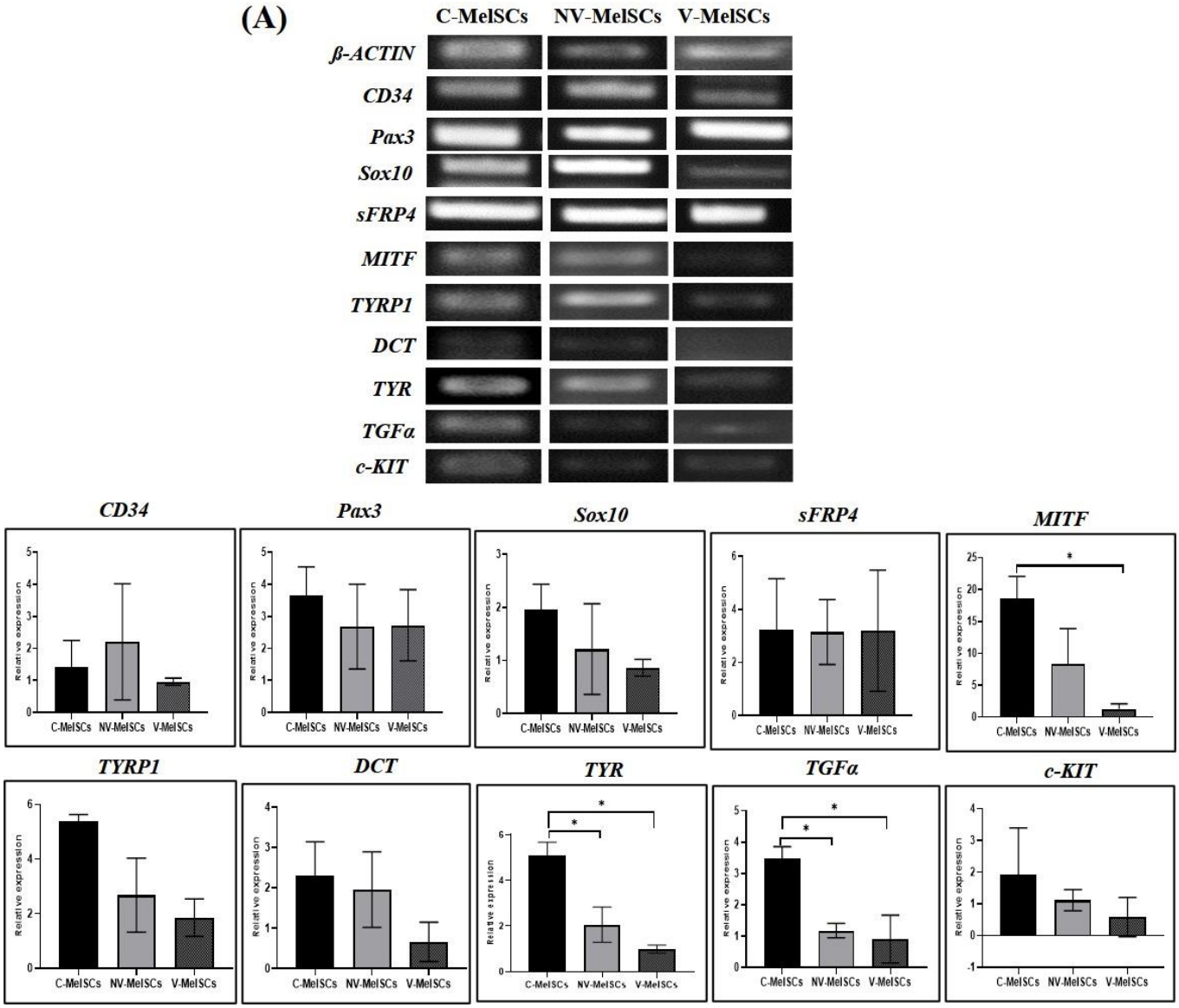

Figure 5. RT-PCR and qPCR Analysis of Selected Genes in MelSCs. **(A)** RT-PCR analysis of selected genes detected as a single fragment at the expected base pairs; **(B)** qPCR results of selected genes. Relative expression of Cluster of differentiation 34 (*CD34*), Paired box 3 (*Pax3*), SRY-box transcription factor 10 (*Sox10*), secreted frizzled-related protein 4 (*sFRP4*), Melanocyte inducing transcription factor (*MITF*), Tyrosinase related protein 1 (*TYRP1*), Dopachrome tautomerase (*DCT*), Tyrosinase (*TYR*), Transforming growth factor – α (*TGFα*), and *c-KIT* transcripts in C-MelSCs, NV-MelSCs, and V-MelSCs. * represents significant differences at $P<0.05$. Data are expressed as mean ± standard deviation (SD).

from the MelSC reservoir in the hair follicle bulge.[19,20] It was reported that 63% of MelSCs exist in the follicular regions of vitiliginous skin.[21] However, it has not been well understood whether the disease progression has an impact on the potency and stemness of MelSCs.

In this study, MelSCs from lesioned and non-lesioned regions of vitiligo patients were compared for their cellular and molecular characteristics with MelSCs derived from normal subjects. Earlier studies used the suspension of outer root sheath cells as a source of MelSCs. This technique requires a substantial number of hair follicles which was not feasible from a 2×2 mm punch biopsy of vitiligo skin. Therefore, we extracted hair follicles by dispase digestion and directly used them as explants. It required evidently longer for the initial release of cells from the hair follicle explants of vitiligo patients than normal subjects. This observation suggests an impact of disease manifestation on the MelSC reservoir of vitiligo hair follicles.

Primary cultures obtained had a heterogeneous population of cells consisting of spindle-shaped dermal fibroblasts, squamous keratinocytes, and possibly mature melanocytes. However, due to their low proliferative capacity and less adherence, keratinocytes and differentiated melanocytes were almost lost at the third passage. Although fibroblasts continued to grow, their proliferation was limited owing to the components of the culture medium. MEM-α was used as the basal media supplemented with FBS, HMGS, and EGF. HMGS consists of basic fibroblast growth factor (bFGF),

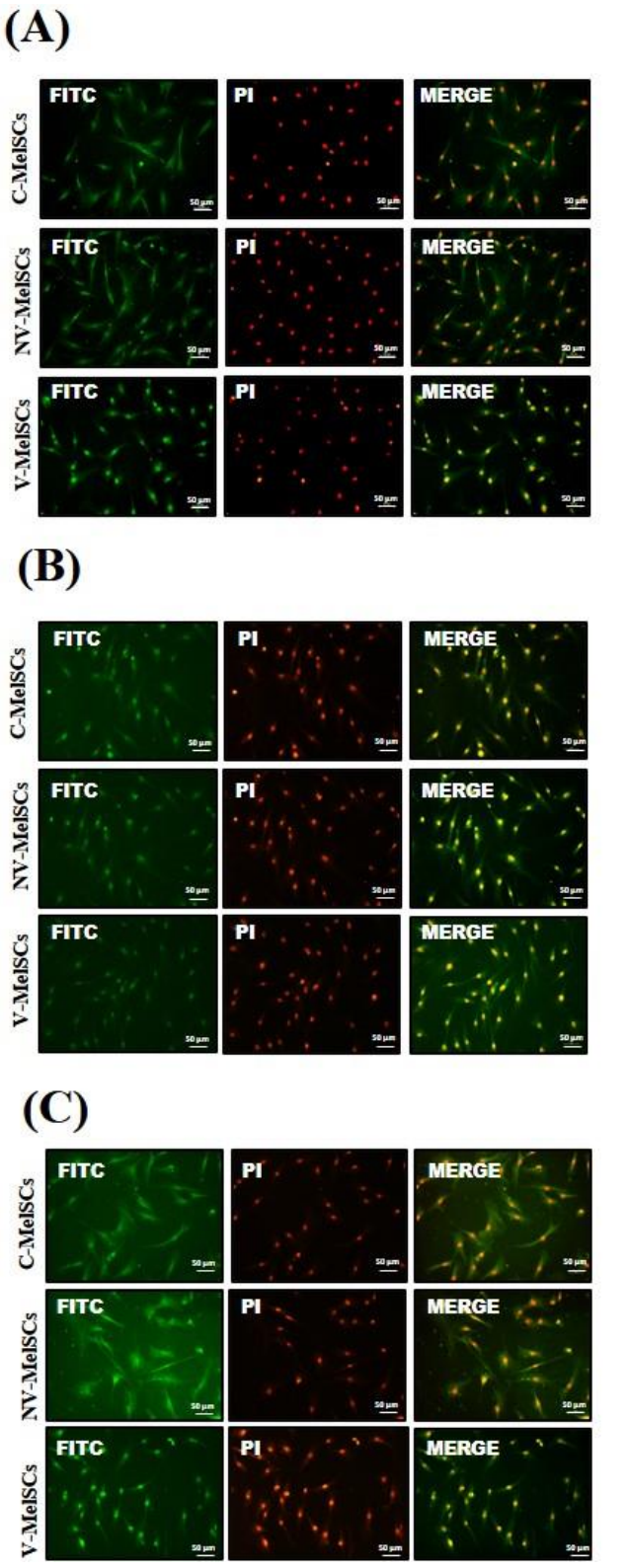

Figure 6. Immunofluorescence Analysis of Selected Markers in MelSCs. MelSCs of all groups were stained positive for (A) CD34, (B) MITF, and (C) TYRP1 (20x). FITC-conjugated (green) secondary antibody was used. Nuclei were counterstained with PI (red). Scale bar = 50 µm.

hydrocortisone, phorbol myristate acetate (PMA), bovine insulin and transferrin, and bovine pituitary extract, all of which are essential components for melanocyte growth and maintenance.[22,23] PMA retards the growth of keratinocytes and, at the same time, induces dendritogenesis and melanocyte - lineage cell proliferation.[24,25] bFGF and EGF have mitogenic properties and promote melanocyte and melanocyte precursors proliferation.[24,26] The concentration of FBS and HMGS in the medium was higher in the primary cultures merely to promote the successful release of cells from the explant and reduced in the later passages to impede differentiation of melanocyte precursor cells and fibroblasts in culture.

MelSCs from adult human hair follicle bulges were first isolated by Tobin et al. in 1995 and were described as "amelanotic melanocytes" due to their inability to produce melanin while retaining most other melanocyte-like characteristics.[25] Their morphology was reported to be bipolar, with neuronal shapes with high proliferative capacity and the ability to differentiate into melanin-producing melanocytes when stimulated. In this study, MelSCs did not differ morphologically between the groups and observed bi-, tri-, and multi-polar shapes with prominent nuclei within large cell bodies. They tended to form the typical honeycomb pattern of melanocytes at high confluency. Colony-forming ability, a foremost characteristic of stem cells, provided evidence that the cells indeed had stem-cell-like properties. A higher percentage of cells in the G0/G1 phase of the cell cycle suggested their quiescent phase, and low senescence at later passage further confirmed the "stemness" of MelSCs. Above 90% of viable cells at every passage indicated their high quality and survival. However, the doubling time of both vitiligo groups was longer than controls, indicating a deficit in the proliferative capacity of MelSCs even in the unaffected regions. These results agree with recent immune-histochemical studies that showed poor wound healing and infiltrations of CD8+ cells in the unaffected areas of vitiligo patients, rendering the areas susceptible to prospective immune attack.[27,28]

Molecular characterization of MelSCs was performed by RT-PCR as well as qPCR. Although no specific markers have been established to identify hair follicle-derived MelSCs, the expression of specific stem cell markers has been recognized. *Pax3* and *Sox10* are such markers that play a significant role in maintaining the undifferentiated quiescence state of MelSCs in the hair follicle niche. Pax3 functions as a regulator of MITF, whereby it induces the expression of MITF but simultaneously competes with MITF for an enhancer region required for downstream regulation of melanogenic genes.[29] Hence, its expression is crucial for the repression of melanogenesis and self-maintenance of MelSCs. *Sox10* is a transcription factor typically involved in embryonic developmental stages. However, its functions in adult tissue stem cells have equal standing. *Sox10* expression has been identified in the hair follicle bulge region throughout the hair follicle cycle.[30] This consistent expression of *Sox10* indicates its involvement in preserving stemness. In our

study, MelSCs of all groups expressed *Pax3* and *Sox10*, signifying the undifferentiated status of the cells. Although the expression of *Sox10* in V-MelSCs was low, it did not differ significantly from other cells as evaluated by qPCR.

MITF-positive cells have been identified in the outer root sheath and the follicular bulge epithelium.[31] *MITF* codes for a nuclear transcription factor that plays a crucial role in melanocyte development and maintenance of potency. In this study, downregulation of *MITF* was observed in both vitiligo groups compared to the control, even though a significant difference was only observed with V-MelSCs. As *MITF* has a regulatory function over other melanocyte-related genes, such as *TYRP1* and *DCT*, these too showed an expression loss in the vitiliginous regions, although not significantly.

MelSCs do not express *TYR in vivo*. Conversely, in Western blot results reported previously, melanocyte precursors in culture expressed TYR enzyme if not quantitatively as much as terminal melanocytes.[32] Similar positive expression of the *TYR* gene was obtained in this study by qPCR analysis of cultured MelSCs in all groups. Nevertheless, the expression was markedly lower in both vitiligo groups.

CD34 is a transmembrane protein that has been popularly regarded as a hematopoietic stem cell marker. However, *CD34* is expressed by several other cell types, including vascular endothelial progenitors, epithelial progenitors, muscle satellite cells, and interstitial cells, among others.[33] The hair follicle is an integral part of the epithelial unit, and there is evidence to suggest the presence of *CD34* positive subpopulation of cells in the hair follicle stem cell niche.[34] *CD34* positive hair follicle cells are located in the bulge area and can potentially differentiate into melanin-producing cells.[35,36] In the present study, MelSCs from the hair follicles were positive for *CD34*, as demonstrated by Immuno-fluorescence and RT-PCR. Although the exact function of *CD34* in these cells is not known, it has been shown that *CD34* positivity is predominantly observed during the anagen phase of the hair cycle, indicating that *CD34* functions are related to the proliferation and differentiation of epithelial cells.

The Wnt signaling pathway is decisive for melanocyte differentiation, and it regulates the downstream expression of several melanogenic genes such as *MITF*, *TYR*, and *TYRP1*.[37] Secreted frizzled-related protein-4 (*sFRP4*) is a glycoprotein and a Wnt inhibitor, and it plays a vital role in skin homeostasis.[37] Previous studies showed that *sFRP4* is secreted by neighboring epithelial cells and acts on MelSCs in a paracrine manner to inhibit differentiation and maintain an undifferentiated status.[38] However, in our findings, MelSCs of all groups expressed *sFRP4* as analyzed by RT-PCR. This indicates the autocrine role of *sFRP4* on melanocyte progenitor differentiation in the skin.

MelSCs are highly unstable cells that rapidly undergo differentiation in culture, and hence, obtaining a homogeneous population of MelSCs yet remains a challenge. Further molecular characterization, protein expression profile, and proficiency of MelSCs to differentiate into melanin-producing melanocytes need to be performed to confirm the functional identity of these cells.

Conclusion

The findings of the present study indicate that MelSCs residing in hair follicle bulge of vitiligo patients possess altered cellular, potency, and biological characteristics. Our findings emphasize the challenges in designing strategies for repigmentation therapy in vitiligo by autologous transplantation of MelSCs.

Authors' Contributions

All authors contributed equally to the current study.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

The authors are grateful to Nitte (Deemed to be University) for the support.

References

1. Gho CG, Braun JE, Tilli CM, Neumann HA, Ramaekers FC. Human follicular stem cells: their presence in plucked hair and follicular cell culture. Br J Dermatol. 2004;150(5):860-8. doi:10.1111/j.1365-2133.2004.05862.x
2. Nishikawa-Torikai S, Osawa M, Nishikawa SI. Functional characterization of melanocyte stem cells in hair follicles. J Invest Dermatol. 2011;131(12):2358-67. doi:10.1038/jid.2011.195
3. Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, Moriyama M, et al. Dominant role of the niche in melanocyte stem-cell fate determination. Nature 2002; 416:854-60. doi:10.1038/416854a
4. Le Poole IC, Van Den Wijngaard RM, Westerhof W, Das PK. Presence of T cells and macrophages in inflammatory vitiligo skin parallels melanocyte disappearance. Am J Pathol. 1996;148(4):1219-28.
5. Wu J, Zhou M, Wan Y, Xu A. CD8+ T cells from vitiligo perilesional margins induce autologous melanocyte apoptosis. Mol Med Rep. 2013;7(1):237-41. doi:10.3892/mmr.2012.1117
6. Bahadoran P, Ortonne JP. Classification of surgical therapies for vitiligo. Oxford: Blackwell Publishing; 2007;59-68.
7. Kumar A, Mohanty S, Nandy SB, Gupta S, Khaitan BK, Sharma S, et al. Hair & skin derived progenitor cells: In search of a candidate cell for regenerative medicine. Indian J Med Res. 2016;143(2):175-83. doi:10.4103/0971-5916.180205
8. Kumar A, Mohanty S, Sahni K, Kumar R, Gupta S. Extracted hair follicle outer root sheath cell suspension for pigment cell restoration in vitiligo. J Cutan Aesthet Surg. 2013;6(2):121-5. doi:10.4103/0974-2077.112679
9. Mohanty S, Kumar A, Dhawan J, Sreenivas V, Gupta S. Noncultured extracted hair follicle outer root sheath cell

suspension for transplantation in vitiligo. Br J Dermatol. 2011;164(6):1241-6. doi:10.1111/j.1365-2133.2011.10234.x
10. Yonetani S, Moriyama M, Nishigori C, Osawa M, Nishikawa SI. *In vitro* expansion of immature melanoblasts and their ability to repopulate melanocyte stem cells in the hair follicle. J Invest Dermatol. 2008;128 (2):408-20. doi:10.1038/sj.jid.5700997
11. Glover JD, Knolle S, Wells KL, Liu D, Jackson IJ, Mort RL, et al. Maintenance of distinct melanocyte populations in the interfollicular epidermis. Pigment Cell Melanoma Res. 2015;28(4):476-80. doi:10.1111/pcmr.12375
12. Joshi SS, Tandukar B, Pan L, Huang JM, Livak F, Smith BJ, et al. CD34 defines melanocyte stem cell subpopulations with distinct regenerative properties. PLoS Genet. 2019; 15(4):e1008034. doi:10.1371/journal.pgen.1008034
13. O'Reilly-Pol T, Johnson SL. Kit signaling is involved in melanocyte stem cell fate decisions in zebrafish embryos. Development. 2013;140(5):996-1002. doi:10.1242/dev.088112
14. Allen JR, Skeath JB, Johnson SL. GABA-A receptor and mitochondrial TSPO signaling act in parallel to regulate melanocyte stem cell quiescence in larval zebrafish. Pigment Cell Melanoma Res. 2020;33(3):416-25. doi:10.1111/pcmr.12836
15. Zhou X, Ma Y, Liu F, Gu C, Wang X, Xia H, et al. Melanocyte chitosan/gelatin composite fabrication with human outer root sheath-derived cells to produce pigment. Sci Rep. 2019;9(1):5198. doi:10.1038/s41598-019-41611-5
16. Morita R, Sanzen N, Sasaki H, Hayashi T, Umeda M, Yoshimura M, et al. Tracing the origin of hair follicle stem cells. Nature. 2021;594(7864):547-52. doi:10.1038/s41586-021-03638-5
17. Ernfors P. Cellular origin and developmental mechanisms during the formation of skin melanocytes. Exp Cell Res. 2010;316(8):1397-407. doi:10.1016/j.yexcr.2010.02.042
18. Meyer KC, Klatte JE, Dinh HV, Harries MJ, Reithmayer K, Meyer W, et al. Evidence that the bulge region is a site of relative immune privilege in human hair follicles. Br J Dermatol. 2008;159(5):1077-85. doi:10.1111/j.1365-2133.2008.08818.x
19. Goldstein NB, Koster MI, Hoaglin LG, Spoelstra NS, Kechris KJ, Robinson SE, et al. Narrow band ultraviolet B treatment for human vitiligo is associated with proliferation, migration, and differentiation of melanocyte precursors. J Invest Dermatol. 2015;135(8):2068-76. doi:10.1038/jid.2015.126
20. Goldstein NB, Koster MI, Hoaglin LG, Wright MJ, Robinson SE, Robinson WA, Roop DR, Norris DA, Birlea SA. Isolating RNA from precursor and mature melanocytes from human vitiligo and normal skin using laser capture microdissection. Exp Dermatol. 2016;25 (10):805-11. doi:10.1111/exd.13072
21. Seleit I, Bakry OA, Abdou AG, Dawoud NM. Immunohistochemical study of melanocyte–melanocyte stem cell lineage in vitiligo; A clue to interfollicular melanocyte stem cell reservoir. Ultrastruct Pathol. 2014; 38(3):186-98. doi:10.3109/01913123.2013.870274
22. Hirobe T, Hasegawa K, Furuya R, Fujiwara R, Sato K. Effects of fibroblast-derived factors on the proliferation and differentiation of human melanocytes in culture. J Dermatol Sci. 2013;71(1):45-57. doi:10.1016/j.jdermsci.2013.03.012
23. Hirobe T, Shibata T, Fujiwara R, Sato K. Platelet-derived growth factor regulates the proliferation and differentiation of human melanocytes in a differentiation-stage-specific manner. J Dermatol Sci. 2016;83(3):200-9. doi:10.1016/j.jdermsci.2016.05.010
24. Zhu WY, Zhang RZ, Ma HJ, Wang DG. Isolation and culture of amelanotic melanocytes from human hair follicles. Pigment Cell Res. 2004;17(6):668-73. doi:10.1111/j.1600-0749.2004.00190.x
25. Tobin DJ, Colen SR, Bystryn JC. Isolation and long-term culture of human hair-follicle melanocytes. J Invest Dermatol. 1995;104(1):86-9. doi:10.1111/1523-1747.ep12613573
26. Hirobe T. Endothelins are involved in regulating the proliferation and differentiation of mouse epidermal melanocytes in serum-free primary culture. J Investig Dermatol Symp Proc. 2001;6(1):25-31. doi:10.1046/j.0022-202x.2001.00001.x
27. Gupta A, Chauhan A, Priya A, Mantri B, Wadhokar M, Dalave K, et al. Lesional skin in vitiligo exhibits delayed in vivo reepithelialization compared to the nonlesional skin. Wound Repair Regen. 2020;28(3):307-14. doi:10.1111/wrr.12798
28. Aslanian FM, Noé RA, Antelo DP, Farias RE, Das PK, Galadari I, et al. Immunohistochemical findings in active vitiligo including depigmenting lesions and non-lesional skin. Open Dermatol J. 2008;2(1):105-10. doi:10.2174/1874372200802010105
29. Qiu W, Chuong CM, Lei M. Regulation of melanocyte stem cells in the pigmentation of skin and its appendages: Biological patterning and therapeutic potentials. Exp Dermatol. 2019;28(4):395-405. doi:10.1111/exd.13856
30. Jing J, Xu P, Xu JL, Ding YX, Yang XS, Jin XQ, et al. Expression and localization of Sox10 during hair follicle morphogenesis and induced hair cycle. Int J Med Sci. 2021;18(15):3498-3505. doi:10.7150/ijms.60728
31. Gleason BC, Crum CP, Murphy GF. Expression patterns of MITF during human cutaneous embryogenesis: evidence for bulge epithelial expression and persistence of dermal melanoblasts. J Cutan Pathol. 2008;35(7):615-22. doi:10.1111/j.1600-0560.2007.00881.x
32. Wang D, Xu X, Ma H, Yue X, Li C, Zhu W. Optimization of the method for the culture of melanocyte precursors from hair follicles and their activation by 1, 25-dihydroxyvitamin D3. Exp Ther Med. 2013;6(4):967-72. doi:10.3892/etm.2013.1252
33. Sidney LE, Branch MJ, Dunphy SE, Dua HS, Hopkinson A. Concise review: evidence for CD34 as a common marker for diverse progenitors. Stem Cells. 2014;32(6): 1380-9. doi:10.1002/stem.1661
34. Jiang S, Zhao L, Purandare B, Hantash BM. Differential expression of stem cell markers in human follicular bulge and interfollicular epidermal compartments. Histochem Cell Biol. 2010;133:455-65. doi:10.1007/s00418-010-0684-z
35. Ali IQ, Salih SM, Abdulla GA. Differentiation of CD34+ human hair follicle stem cells into functional melanocytes. IOP Conf Ser Mater Sci Eng. 2018;454:1-10. doi:10.1088/1757-899X/454/1/012013
36. Joshi SS, Tandukar B, Pan L, Huang JM, Livak F, Smith BJ, et al. CD34 defines melanocyte stem cell subpopulations with distinct regenerative properties. PLoS genet. 2019;15(4):e1008034. doi:10.1371/journal.pgen.1008034
37. Li H, Hou L. Regulation of melanocyte stem cell behavior by the niche microenvironment. Pigment Cell Melanoma Res. 2018;31(5):556-69. doi:10.1111/pcmr.12701
38. Guo H, Lei M, Li Y, Liu Y, Tang Y, Xing Y, et al. Paracrine secreted frizzled-related protein 4 inhibits melanocytes differentiation in hair follicle. Stem Cells Int. 2017;2017:2857478. doi:10.1155/2017/2857478